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Characterization and partial purification of liver glucose transporter GLUT2

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Abstract

GLUT2, the major facilitative glucose transporter isoform expressed in hepatocytes, pancreatic β-cells, and absorptive epithelial cells, is unique not only with its low affinity and broad substrate specificity as a glucose transporter, but also with its implied function as a glucose-sensor. As a first essential step toward structural and biochemical elucidation of these unique, GLUT2 functions, we describe here the differential solubilization and DEAE-column chromatography of rat hepatocyte GLUT2 protein and its reconstitution into liposomes. The reconstituted GLUT2 bound cytochalasin B in a saturable manner with an apparent dissociation constant (K_d) of 2.3×10^{-6} M and a total binding capacity (B_T) of 8.1 nmol per mg protein. The binding was completely abolished by 2% mercury chloride, but not affected by cytochalasin E. Significantly, the binding was also not affected by 500 mM D-glucose or 3-*O*-methyl D-glucose (3OMG). The purified GLUT2 catalyzed mercury chloride-sensitive 3OMG uptake, and cytochalasin B inhibited this 3OMG uptake. The inhibition was dose-dependent with respect to cytochalasin B, but was independent of 3OMG concentrations. These findings demonstrate that our solubilized GLUT2 reconstituted in liposomes is at least 60% pure and functional, and that GLUT2 is indeed unique in that its cytochalasin B binding is not affected by its substrate (D-glucose) binding. Our partially purified GLUT2 reconstituted in vesicles will be useful in biochemical and structural elucidation of GLUT2 as a glucose transporter and as a possible glucose sensor.

Keywords: GLUT2; Purification; Detergent solubility; Reconstitution; Cytochalasin B; Hepatocyte

1. Introduction

A family of intrinsic membrane proteins known as facilitative glucose transporters catalyzes the uptake and release of glucose and other selected sugars in animal cells [1,2]. Five functional isoforms (GLUT1–5) are known in this family, which differ significantly in terms of tissue distribution, regulation, transport kinetics and substrate specificity. GLUT1 [3] is ex-

Abbreviations: CB, cytochalasin B; CE, cytochalasin E; DEAE, diethylaminoethylcellulose; DM, dodecylmaltoside; DOC, sodium deoxycholate; DTAB, dodecyltrimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid; OG, octylglucoside; 3OMG, 3-*O*-methyl D-glucose; SDS, sodium dodecyl sulfate; SML, sucrose monolaurate; W-1, polyoxyethylene ether w-1; $C_{12}E_8$, polyoxyethylene 8 lauryl ether

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pressed widely in many cells and is particularly abundant in human erythrocytes and transformed cells. GLUT2 is selectively expressed in liver cells, pancreatic β-cells as well as intestinal and renal epithelial cells [4–6]. GLUT3 is found typically in brain tissues [7], whereas GLUT4 is expressed in muscle and fat cells, the classical insulin-target cells [8]. Of these, GLUT1 is the only isoform that has been available as a pure and functional protein, allowing direct biochemical and structural elucidation of this isoform.

GLUT2 function in intact cells and isolated membranes has been studied extensively [6,9–11], revealing a number of features unique to this isoform. Its transport affinity to D-glucose (K_m of 15–40 mM) is an order of magnitude lower than that of other isoforms in this family (K_m of 1–5 mM). It also shows a low affinity to cytochalasin B, a potent inhibitor of all GLUT isoforms (with a K_i of 2 μM or higher) [10,12], again distinguishing itself from other isoforms (K_i of 0.1–0.3 μM) [13,14]. Although controversial [10,12], this GLUT2 inhibition by cytochalasin B was shown to be not reversed by an excess of D-glucose [15,16]. This is in contrast to GLUT1 [13] and GLUT4 [14], where the inhibition by cytochalasin B is readily reversed by D-glucose. Equilibrium binding studies also revealed a low affinity of GLUT2 to cytochalasin B and a non-competitive mode of interaction with respect to D-glucose [15,16]. This is in contrast to GLUT1 [13] and GLUT4 [14] where glucose readily inhibits cytochalasin B binding. It is important to note, however, that GLUT2 expressed in *Xenopus* oocytes showed a transport K_m that is not much different from the other isoforms [17], suggesting that some of these GLUT2 characteristics observed in cells may not be the property of GLUT2 protein itself, but mediated by a cell factor or factors. GLUT2 is also unique in that it accepts fructose as a substrate [18]. Structural determinants for some of these GLUT2-specific properties have been unraveled by recent molecular biological approaches [19–22].

GLUT2 is also unique among the glucose transporter isoforms in that it is believed to participate in glucose sensing or signaling. Thus, GLUT2 is thought to be a glucose sensor for the glucose-stimulated insulin secretion in rodent pancreatic beta cells [23–26]. In bacteria, a glucose transporter that is highly analogous to GLUT2 was shown to function as a glucose receptor that initiates a signal cascade for gene regulation [27]. Furthermore, GLUT2 may be a specific target molecule for streptozotocin (STZ)-induced toxicity of beta cells in rodents [28–30]. Molecular mechanisms underlying these additional, apparently transport activity-independent, GLUT2 functions are not known.

In the present study, we have characterized rat hepatocyte GLUT2 for its differential solubility, partial purification in DEAE-column chromatography, and functional reconstitution into liposomes. We show that our partially purified GLUT2 is functional in that it possesses a mercury chloride-dependent and cytochalasin E-independent cytochalasin B binding activity and catalyzes stereospecific and cytochalasin B-sensitive glucose uptake. We also demonstrate that the reconstituted GLUT2 shows significantly lower affinities to D-glucose and cytochalasin B compared to other isoforms. Our GLUT2 preparation will be useful for direct elucidation of the biochemical and structural bases of GLUT2 function as a transporter as well as a presumed glucose sensor.

2. Materials and methods

2.1. Materials

Octylglucoside (OG), sucrose monolaurate (SML) and dodecylmaltoside (DM) were obtained from Boehringer Mannheim. Sodium deoxycholate (DOC), CHAPS, dodecyltrimethylammonium bromide (DTAB), Triton X-100, $C_{12}E_8$, brij-35, brij-721, polyoxyethylene ether w-1 (W-1), cytochalasins B and E, collagenase and protease inhibitor cocktail were from Sigma. [^{3}H]Cytochalasin B and [^{14}C]3-*O*-methyl D-glucose (3OMG) were purchased from Amersham. BSA was from Armour Pharmaceuticals and anti-GLUT2 antibody was purchased from East Acres Biological.

2.2. Solutions

Hank's buffer was made of 125 mM NaCl, 4.9 mM KCl, 0.73 mM $MgSO_4$, 0.32 mM Na_2HPO_4, 0.40 mM KH_2PO_4, 1.15 mM $CaCl_2$, 4.2 mM $NaHCO_3$, and 5.5 mM glucose. Ca^{2+}-free Hanks' buffer contained

137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.35 mM Na_2HPO_4, 0.444 mM KH_2PO_4, 0.5 mM EGTA, 4.2 mM $NaHCO_3$, and 5.5 mM glucose. Krebs–Ringer phosphate buffer was made of 128 mM NaCl, 5.2 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, and 10 mM Na_2HPO_4 (pH 7.4). Tris-elution buffer was made of 50 mM Tris–HCl (pH 7.4), 1 mM DTT, 0.1 mM EDTA and a detergent (1% W-1, 0.2% SML, 0.1% DM, or 1% OG). Phosphate elution buffer included 10 mM Na_2HPO_4 (pH 7.5), 1 mM DTT, 0.1 mM EDTA and a detergent (1% W-1, 0.2% SML, 0.1% DM, or 1% OG). Dialysis buffer A was made of 10 mM Na_2HPO_4 (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 150 mM NaCl and 500 mM D-glucose. Dialysis buffer B contained 10 mM Na_2HPO_4 (pH 8.0), 1 mM DTT and 0.1 mM EDTA.

2.3. Isolation of hepatocytes and hepatocyte membranes

Hepatocytes were isolated free of erythrocyte contamination from Sprague–Dawley rats, 170–220 g body weight, as described previously [31]. Briefly, rat liver was perfused for 20 min with 400 ml of Ca^{2+}-free Hanks' buffer solution until liver becomes cream colored, then for 8 min with 100 ml of Hanks' buffer solution containing collagenase (Sigma C-5138, 100 mg/150 ml). Liver was removed, gently dispersed in 50 ml Hanks-collagenase solution, and shaken in a water bath at 37°C for 10 min. The mixture was filtered through nylon mesh and the filtrate was spun at 1000 rpm for 3 min at 4°C using Sorvall SS-34 rotor. Isolated hepatocytes were resuspended in cold Hanks buffer, let settle for 20 min and the supernatant was removed. This washing procedure was repeated two more times to ensure complete removal of contaminating erythrocytes. Cells were then resuspended in 50 mM Tris buffer (pH 7.4), (10 ml for cells from one liver) and stored at 4°C overnight, if needed. Suspension of hepatocytes was centrifuged at $10\,000 \times g$ (Sorvall) at 4°C for 10 min. The packed cells (1 ml) were homogenized in 5 ml buffer containing 10 mM HEPES (pH 7.5) 0.25 M sucrose and protease inhibitor cocktail (1 ml, general use) at 2000 rpm with three or four strokes. The homogenate was centrifuged at $200\,000 \times g$ for 180 min to obtain total membrane pellet.

2.4. Extraction with EDTA

Total membrane pellet (750–800 mg protein) was dispersed and stirred in 1 mM EDTA in final volume of 1500 ml (pH 7.5) on ice for 30 min and then centrifuged at 12 000 rpm in a GSA rotor for 60 min. The pellet was dispersed into 1000 ml of 0.1 mM EDTA (pH 10.2) and centrifuged right away followed by a wash with a buffer (250 ml) to be used (EDTA-pellet) in the next step.

2.5. Extraction with detergents

A suspension of EDTA-pellet (50–75 mg protein) was stirred at 4°C for 30 min with 10 mM Tris buffer (pH 7.5) containing 1 mM DTT, 100 μl of protease inhibitor cocktail and specified detergent to 1% final concentration unless indicated otherwise. The mixture was centrifuged at 45 000 rpm with Ti 50.2 rotor (Beckman ultracentrifuge L5-50) and the pellet removed. GLUT2 was detected by Western blots using rabbit anti-GLUT2 antibody and chemiluminescence visualization protocol (Amersham).

2.6. DEAE-column chromatography

Detergent extract of EDTA-pellet (50–60 mg protein in 15 ml) was introduced on to microgranular diethylaminoethylcellulose (DE 52, Whatman) column (1×10 cm bed) equilibrated in elution buffer (Tris-elution buffer, or phosphate elution buffer) containing the detergent in which protein was extracted, at room temperature. Following chase with 20 ml of elution buffer, a sodium chloride gradient in elution buffer was applied as specified.

2.7. Reconstitution into liposomes

Partially purified GLUT2 samples used for reconstitution include EDTA pellet and each of the three NaCl eluates of DEAE column chromatography in OG. The NaCl eluates were concentrated using 10 K Centricon at 4°C, and made 500 mM with respect to D-glucose prior to reconstitution. Soybean lipids (20 mg) dispersed in 0.5 ml of 2% sodium cholate in dialysis buffer A were mixed with partially purified GLUT2 solution in 1% OG (5 ml, 1.0 mg protein). After stirring at 4°C for 30–60 min, the mixture was

diluted with detergent-free dialysis buffer A without glucose to 18 ml, and centrifuged at 45 000 rpm with Ti 50.2 rotor for 60 min in an ultracentrifuge to collect pellet (reconstituted GLUT2 in liposomes). From DM-solubilized GLUT2, dried soybean lipids (20 mg) were dispersed in 0.5 ml of 2% sodium cholate in dialysis buffer A, mixed with GLUT2 in DM (0.1%, 0.5 ml) and concentrated to 2 mg/ml. After stirring at room temperature for 60 min, the mixture was dialyzed against dialysis buffer A overnight at room temperature. The mixture was further dialyzed for 48 h at 4°C against dialysis buffer B with one change of buffer. Reconstituted GLUT2 in vesicles was collected by centrifugation (260 000 × *g* for 60 min).

2.8. Gel electrophoresis and Western blot

Proteins were separated in SDS–PAGE according to Laemmli [32]. Proteins in gels were transferred to Immobilon-P membrane and GLUT2 was visualized by immunoblot as described [31] using rabbit polyclonal antisera specific to GLUT2 (1/500 dilution). Relative immunoblot intensities were quantitated by densitometry using an analytical scanning system (Molecular Dynamics, Sunnyvale, CA).

2.9. Cytochalasin B binding assay

Equilibrium binding of cytochalasin B to GLUT2 reconstituted in vesicles was measured by a centrifugation method using [^{3}H]cytochalasin B as a tracer [13]. Briefly, GLUT2 vesicles were incubated with a fixed tracer amount of [^{3}H]cytochalasin B (0.1 μCi/ml) at a specified concentration of cold cytochalasin B, with or without cytochalasin E or D-glucose, in 1 ml (final volume) of a 10 mM Tris-HCl buffer (pH 7.4) for 30 min at room temperature, and centrifuged at 260 000 × *g* at 4°C for 60 min. Pellets and supernatants were recovered and their radioactivities were measured, from which the amounts of bound ligands were calculated in percent of the total ligands in the assay mixture. Binding data were analyzed in Scatchard plots [33].

2.10. Glucose transport assay

Equilibrium exchange flux of 3-*O*-methyl D-glucose across GLUT2-containing vesicles was measured by millipore filtration method [34] as modified [35], using [^{14}C] 3-*O*-methyl D-glucose as tracer. Briefly, GLUT2-containing vesicles were prepared by reconstitution (see above) from the 1000 mM NaCl eluate of DEAE chromatography in OG. Time course of tracer equilibrium exchange uptake of 3OMG was measured by arresting flux at specified time points using prechilled, 2% mercury chloride solution. Millipore filters (0.22 μm pore, GSWP) and an Amicon vacuum filtration manifold (VFMI, Amicon, Danvers, MA) were used for separation of vesicles from suspension medium. Ten microliters of GLUT2 vesicle suspension containing approximately 0.4 μg of protein and 20 μg of lipid was used for each time point. The equilibrium intravesicular space of the GLUT2-vesicle suspension used for transport assay was approximately 0.03% of assay suspension.

2.11. Protein determination

Bradford [36] dye binding assay (Bio-Rad) was used for protein determination in OG, DM, or SML solutions. For dilute protein solutions in W-1, the detergent was removed by acetone extraction of lyophilized protein residue before SDS gel electrophoresis prior to protein determination by Lowry et al. [37]. Protein in the pellet was assayed according to Peterson [38].

3. Results

3.1. Differential solubilization of GLUT2 by EDTA and detergents

The isolated rat hepatocytes used in the present study were practically free of erythrocytes with no detectable GLUT1 in immunoblots (not shown) [31]. Typically, 700–900 mg of total membrane protein was obtained from each rat (170–220 g body weight) liver. Total membranes were first subjected to EDTA-alkaline treatment to remove extrinsic membrane proteins. A 30 min incubation with 1 mM EDTA (pH 7.5) followed by a brief wash with 0.1 mM EDTA (pH 10.2) removed 70–80% of proteins from total membranes with less than 10% of GLUT2 loss (Fig. 1 and Table 1). Treatment with

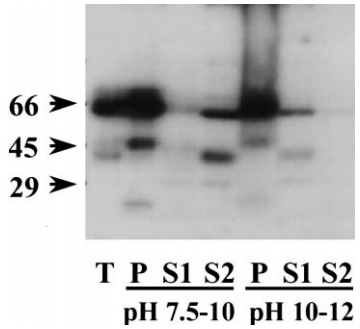

Fig. 1. Enrichment of GLUT2 in EDTA-insoluble membrane fraction. Hepatocyte total membranes (50 mg protein) were suspended and stirred in 100 ml of buffer containing 1 mM EDTA (pH 7.5 or 10.0) for 30 min, and insoluble pellet was separated from supernatant (S1) by centrifugation (185 000 × *g* for 30 min at 4°C). This pellet was again treated with 100 ml of 0.1 mM EDTA (pH 10.2 or 12.0) and the pellet (P) separated similarly from supernatant (S2). The final pellets and supernatants were subjected to immunoblot for GLUT2 quantitation as described in Section 2. Lanes are total membranes (T, 50 μg protein), final pellet (P, 25 μg protein) and two supernatants (S1 and S2, 50 μg protein each) for each set of pH treatment as specified underneath.

pH 10 followed by a pH 12 wash resulted in a slight increase in protein extraction (not illustrated) with a similar GLUT2 recovery, but with significant GLUT2 aggregation (Fig. 1). Western blot analyses of these EDTA-pellets revealed a broad, 60-kDa band as the major GLUT2 immunoreactive protein (Fig. 1). They also revealed a minor immunostaining band at either the 45- or 40-kDa position (Fig. 1), whose identity was not immediately clear.

Further purification of GLUT2 requires its solubilization in appropriate, non-ionic detergents. A large number of detergents were tested for their ability to solubilize GLUT2 from EDTA-extracted membranes (illustrated in Fig. 2 and summarized in Table 1). Unexpectedly, detergent solubility of liver GLUT2 was found to be drastically different from that of erythrocyte GLUT1. OG (Fig. 2A) which effectively solubilizes GLUT1 in human erythrocyte membranes [39,40] was unable to solubilize GLUT2 from hepatocyte membranes. $C_{12}E_8$ and Triton X-100, which solubilize erythrocyte GLUT1 more than 90%, solublized GLUT2 to a significant extent, but with an extensive aggregation. GLUT2 solubility in some detergents was found to be pH-dependent (not illustrated). SML solubilized practically no GLUT2 at pH 6.5, while it solubilized 55 and 65% of GLUT2 at pH 7.5 and 9.0, respectively.

Two non-ionic detergents, DM and W-1, on the other hand, solubilized GLUT2 quite effectively and, to some degree, selectively (Fig. 2 and Table 1). Thus, 1% DM solubilized 80–90% of GLUT2 with only 30–35% of the protein. DM 0.1 and 0.02% solu-

Table 1
Differential solubilization of GLUT2 and proteins by EDTA and by various detergents

Extracting reagents	Protein solublized (%)	GLUT2 solublized (%)
EDTA, 1 mM (pH 7.5)	32 ± 5	5
EDTA, 0.1 mM (pH 10.2)	72 ± 2	10
EDTA, sequential treatment	83 ± 3	10
OG	52 ± 5	0
$C_{12}E_8$	53 ± 6	50*
CHAPS	55 ± 7	50**
DM	33 ± 4	85
SML	50 ± 7	70
Triton X-100	66 ± 8	75***
Brij-35	45 ± 4	25
Brij-721	37 ± 7	20
W-1	32 ± 4	90
DOC	71 ± 8	40***
SDS	97 ± 4	100
DTAB	85 ± 5	80

Each treatment was for 30 min at 4°C except for W-1, which was at room temperature. Fifty milligrams of total membrane protein (for EDTA extraction) or EDTA-washed pellets (for detergent extraction) was used. Protein was assayed either by Lowry [37] or by Bradford [36]. Values are the mean ± S.D. of three to five independent experiments. GLUT2 was estimated based on immunoblot intensities. Some detergent extracts showed GLUT2 aggregation to a varying degree as high as 30% (*), 60% (**), and 90% (***).

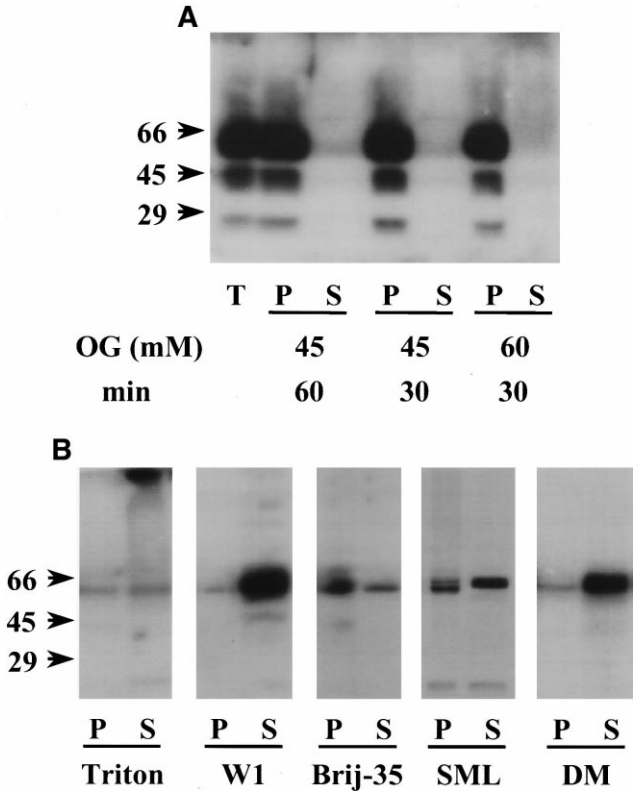

Fig. 2. Effect of detergents on GLUT2 in EDTA (pH 7.5–10.2)-washed membranes (EDTA-pellet). (A) OG treatment under various conditions. EDTA-pellet (50 mg protein) was stirred in 22 ml of 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM DTT, 0.1 mM EDTA, 100 μl of protease inhibitor cocktail and 45 or 60 mM OG, at 4°C for 30 or 60 min. After centrifugation (185 000×*g*, for 60 min at 4°C), the pellets and supernatants were immunoblotted by applying 50 μg protein in each lane. Samples are EDTA-pellet prior to OG treatment (T), and pellet (P) and supernatant (S) after treatment at OG concentration and duration specified underneath. (B) Treatment with Triton X-100 (Triton), W-1 (W1), Brij-35, SML and DM at 1% each, for 30 min, at 4°C except for W-1 which was used at room temperature. Pellets (P) and supernatants (S) were subjected to immunoblots. Each lane contained 50 μg protein for Triton, W-1 and DM, and 25 μg for Brij-35 and SML. Similar results were observed in three additional experiments.

bilized GLUT2 at approximately 70 and 20%, respectively. W-1, a polyoxyethylene ether detergent, also solubilized 90–95% of GLUT2 with only 32% of the proteins. Ionic detergents, SDS and DTAB, solubilized most of the GLUT2 along with most of the protein, as expected. Sequential treatments with a non-solubilizing detergent followed by solubilizing detergents did not give any significant improvement in GLUT2 solubilization or its purification (not illustrated).

3.2. Partial purification of GLUT2 by DEAE-cellulose column chromatography

EDTA-washed, human erythrocyte GLUT1 solubilized in OG or Triton X-100 is readily purifiable as a flow-through in DEAE-cellulose column chromatography [34,40,41]. Unlike GLUT1, GLUT2 in EDTA-washed pellet solubilized in either DM (Fig. 3) or W-1 (Fig. 4) was retained on the anion exchange resin and was not released even after an extensive wash that eluted 45–50% of the protein. Subsequent elution with an increasing concentration of NaCl, however, released GLUT2 (Figs. 3 and 4). Upon the chromatography in DM (0.1%, pH 7.4), GLUT2 was eluted at two distinct salt concentrations, one at 40–50 mM NaCl and the other at 200 mM NaCl concentration (Fig. 3B). At 40 mM NaCl concentration, GLUT2 was eluted gradually, suggesting involvement of hydrophobic interactions. It could also be due to slow dissolution of released GLUT2, or its slow release, or ionic heterogeneity. The 200 mM NaCl fraction, on the other hand, eluted as a sharp peak and contained 30–40% of the protein with 20–30% of the GLUT2. The 40 mM NaCl fraction shows 20–22-fold overall purification. This fraction could further be resolved; as high as 60% of its GLUT2 could be collected (in fraction numbers 12–35 in Fig. 3B) with less than 1% of its protein, thus giving a 60–70-fold purification with at least 30% GLUT2 recovery.

DEAE-cellulose column chromatography of W-1-solubilized EDTA-pellets in the presence of OG gave a significantly higher GLUT2 purification (Fig. 4). After applying W-1-solubilized GLUT2, the column was washed extensively with a buffer containing 1.1% OG. This effectively exchanged W-1 with OG, with practically no GLUT2 loss (Fig. 4A). This was followed by stepwise elution with buffers containing 50, 200, and 1000 mM NaCl, which eluted GLUT2 effectively (Fig. 4B).

3.3. Reconstitution of GLUT2 into liposomes

W-1 was found to be very difficult to dialyze out. Methods based on exchange in size-exclusion column

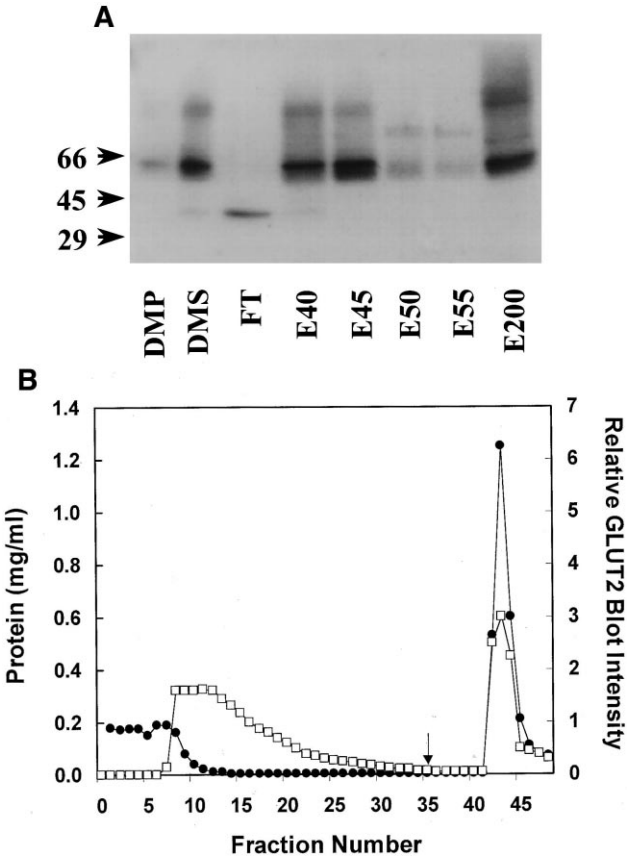

Fig. 3. DEAE cellulose column chromatography of DM-soluble GLUT2 in EDTA-washed membranes. (A) A 50 mg protein equivalent of EDTA (pH 7.5–10.2) washed pellet was suspended in 20 ml of buffer containing 1% DM for 30 min and detergent insoluble and soluble portions were separated by centrifugation (see Section 2). The detergent-soluble portion (20 ml) was applied to DEAE cellulose column (1×10 cm) that was pre-equilibrated with elution buffer (see Section 2) containing 0.1% DM. After collecting flow through, the column was washed by chasing with 20 ml elution buffer, and then eluted in sequence with 15 ml each of the elution buffer containing 40, 45, 50, 55 and 200 mM NaCl. The flow rate was maintained at 1 ml/min throughout. GLUT2 contents in each fraction were measured by immunoblots. Lanes contained 200 μl of flow through plus chase (FT); 40 mM NaCl eluate (E40); 45 mM NaCl eluate (E45); 50 mM NaCl eluate (E50); 55 mM NaCl eluate (E55); and 200 mM NaCl eluate (E200). Also included are 50 μg each of DM-insoluble (DMP) and DM-soluble (DMS) portions of the EDTA-pellet as controls. Protein recoveries in each fraction were 8.4 (FT), 2.8 (E40), 0.9 (E45), 0.52 (E50), 0.30 (E55), and 5.2 (E200) mg, together amounting to 18.2 mg or 36% of the protein in EDTA-pellet. These correspond to 48 (FT), 37 (E40), 12 (E45), 7 (E50), 4 (E55), and 69 (E200) μg protein per lane in immunoblots, respectively. (B) Chromatography was done as described above, except that the sodium chloride elution was carried out first with 40 ml of 40 mM NaCl, followed by 10 ml of 200 mM NaCl. The flow rate was 0.2 ml/min. A 1-ml fraction was collected starting at the application of 40 mM NaCl elution buffer. GLUT2 contents (open squares) were assayed by immunoblots, quantitated by densitometry (see Section 2), and expressed in arbitrary units. Proteins (closed circles) were quantitated by Bradford [36]. Arrow indicates application of 200 mM NaCl elution buffer.

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and adsorption by SM-2 beads were unsuccessful. Anion exchange chromatography on DEAE cellulose in OG, however, succeeded, where GLUT2 was eluted in OG free of W-1 in the presence of 0.2 and 1 mM NaCl as described in Section 2 (Fig. 4). GLUT2 was reconstituted by mixing with lipids and sodium cholate, then diluting with detergent-free buffer (see Section 2) (Fig. 4B). Remarkably, the reconstitution of GLUT2 was highly selective; more than 90% of GLUT2 immunoreactivity and less than 3% of protein in 1000 mM NaCl eluate went to vesicles upon reconstitution (Fig. 4B), resulting in an additional 27–30-fold purification of GLUT2 (Table 2). This unexpected finding may be explained by assuming that the most of the protein species other than GLUT2 in the NaCl eluates are hydrophilic and thus escape incorporation into lipid vesicles.

Table 2
Purification of GLUT2 from rat hepatocyte membranes

Steps	Protein (mg)	GLUT2 yield (%)	Purification
Total membranes	250	100	1
EDTA-pellet	55.1	92.6	4.2
W-1 extract	15.3	78.7	12.8
DEAE-/OG E1000	1.1	58.8	133
Reconstitution	0.04	44.1	2756

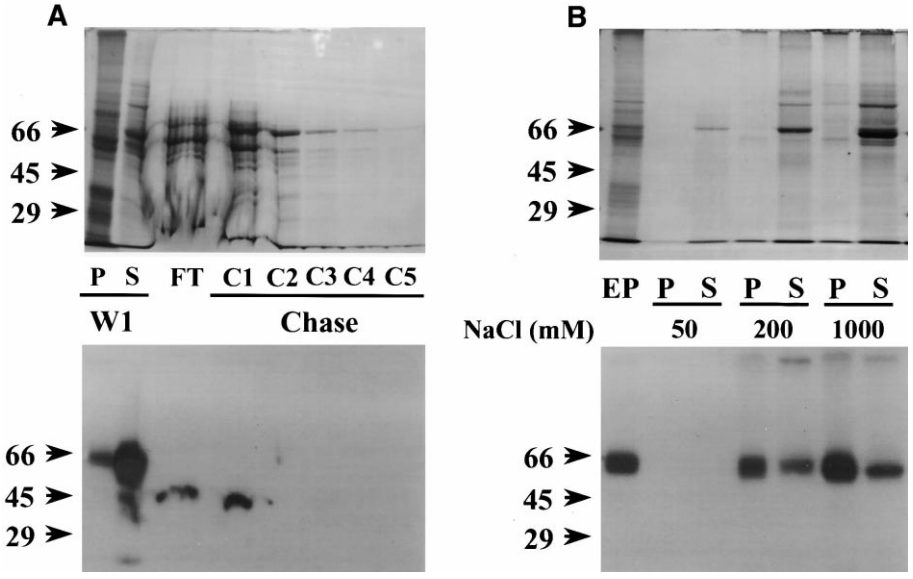

Fig. 4. Chromatography of W-1 solubilized GLUT2 in OG (A) and reconstitution of partially purified GLUT2 into liposomes (B). A 50-mg protein equivalent of EDTA (pH 7.5–10.2) pellet was extracted with 25 ml of 0.5% W-1 for 30 min, and the mixture was centrifuged (185 000×*g* for 30 min) to separate insoluble pellet (P) and supernatant (W-1 extract) (S). W-1 extract (S) was subjected to chromatography using a DEAE column (1×10 cm) that was pre-equilibrated with a Tris–HCl elution buffer containing 1.1% OG (see Section 2). After collecting 25 ml of flow-through (FT), the column was chased with 50 ml of an OG-containing buffer, at 1 ml/min, collecting five 10-ml fractions (C1–C5). This chase effectively exchanged W-1 with OG, without eluting much GLUT2 protein. The column was then eluted with a sodium chloride step gradient, using 15 ml of each of the elution buffer containing 50, 200, and 1000 mM NaCl, at 1 ml per min. These NaCl eluates (50, 200, and 1000, respectively) were used for GLUT2 reconstitution in liposomes as described in Section 2. Both GLUT2 purification and reconstitution were monitored by protein staining with Coomassie blue (upper panels) and GLUT2 immunoblot (lower panels) after SDS gel electrophoresis. (A) Lanes are W-1-insoluble (P, 10 μl or 1/500 of total) and W-1 soluble (S, 50 μl or 1/500 of total) fractions; DEAE flow through (FT, 1 ml or 1/25 of total); and chases (C1–C5, 500 μl or 1/20 of total each). (B) Protein and GLUT2 analyses of the NaCl eluates upon reconstitution (see Section 2). One-tenth of reconstituted vesicles (P) and unreconstituted supernatant (S) for each NaCl eluate were analyzed. Lanes are: EDTA pellet (EP) (26 μg), and one-tenth each of reconstituted-vesicles (P) and the corresponding supernatants (S) for the 50, 200 and 1000 mM NaCl eluates (specified as 50, 200, and 1000, respectively, underneath). Please note that a fixed fraction (rather than fixed amount of protein) of NaCl eluate was loaded in each lane. Protein contents in reconstituted vesicles were approximately 1.1, 2.1, and 4.5 μg for 50, 200 and 1000 mM NaCl eluates, respectively, when 25 mg of total membrane protein was processed for purification and reconstitution.

3.4. Preliminary characterization of partially purified GLUT2 in liposomes

The GLUT2-vesicles purified and reconstituted from the 1000 mM NaCl eluate of DEAE chromatography in the presence of OG as illustrated in Fig. 4B bound cytochalasin B avidly (Fig. 5). The binding was dose-dependent with respect to cytochalasin B. This cytochalasin B binding was not affected at all by the presence of 10^{-4} M cytochalasin E (not illustrated), demonstrating that the binding is not due to possible actin contamination. Significantly, this saturable cytochalasin B binding was completely abolished by 2% mercury chloride (not illustrated), but was not affected at all by the presence of 500 mM D-glucose (Fig. 5) or 3OMG (not illustrated). This demonstrates that the transport substrates do not affect GLUT2 cytochalasin B binding in vitro. Liposomes that did not contain purified GLUT2 also bound cytochalasin B, but the binding was not saturable with increasing cytochalasin B concentrations (Fig. 5). Analyses [33] of the data revealed that our partially purified GLUT2 reconstituted in lipid vesicles typically binds cytochalasin B with an apparent K_d of 2.3 μM and a total capacity (B_T) of 8.1 nmol/mg protein.

The purified GLUT2-vesicles studied above for cytochalasin B binding also showed mercury chloride-sensitive 3OMG uptake. When measured under equilibrium exchange conditions, half-equilibration times

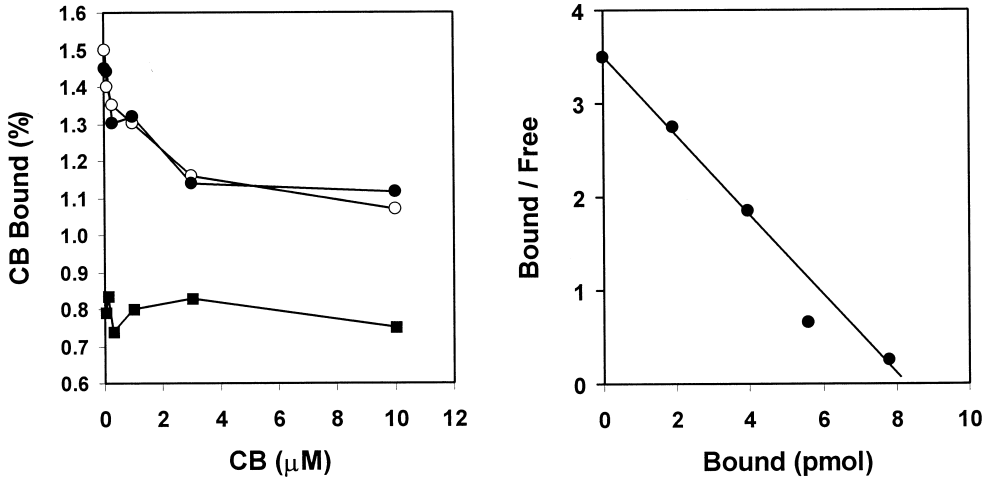

Fig. 5. Cytochalasin B binding to purified GLUT2 reconstituted in vesicles. GLUT2 vesicles reconstituted from the 1000 mM NaCl eluate of DEAE chromatography in OG as illustrated in Fig. 4B was used. Each binding assay mixture contained GLUT2 vesicles corresponding to 0.4 μg protein and approximately 20 μg lipids in 1 ml buffer (see Section 2 for more details). Left panel: percent binding at each CB concentration in binding assay mixture. Data are without (solid circles) and with 500 mM D-glucose (open circles). Also shown are data using liposomes (approximately 15 μg lipids containing no GLUT2) run in parallel (solid squares). Right panel: Scatchard plot of the CB binding data with no additive. Similar results were obtained in three other experiments.

($t_{1/2}$) of approximately 2.5 and 5 min were obtained with 5 and 20 mM of 3OMG, respectively (Fig. 6). In the presence of 2% mercury chloride, the rate of 5 mM 3OMG equilibration by GLUT2-vesicles was greatly reduced showing a $t_{1/2}$ of approximately 21 min (not illustrated). This mercury chloride-insensitive 3OMG flux most likely represents leak flux typical to those of proteoliposomes [34]. Furthermore, cytochalasin B inhibited this 3OMG exchange flux in GLUT2-vesicles in a dose-dependent manner; for 5 mM 3OMG equilibration, the $t_{1/2}$ was approximately 5 and 16 min in the presence of 10^{-6} and 10^{-5} M of cytochalasin B, respectively (Fig. 6).

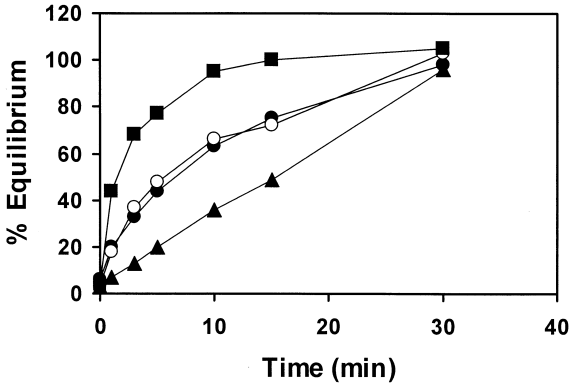

Fig. 6. Cytochalasin B inhibitable 3OMG equilibrium exchange by proteoliposomes containing purified GLUT2. GLUT2 vesicles used were those from the 1000 mM NaCl eluate of DEAE chromatography in OG as illustrated in Fig. 4B. Time course of tracer equilibrium exchange uptake of 3OMG was measured by millipore filtration method [34,35] using [^{14}C]3OMG as a tracer. Flux was arrested at specified time points using prechilled, 2% mercury chloride solution. Data were expressed as % of the complete tracer equilibration as a function of time. A 10-μl of GLUT2 vesicles suspension containing approximately 0.4 μg protein and 20 μg of lipids was used for each time point. Data are for 5 mM (solid squares) or 20 mM (open circles) 3OMG with no CB, and for 5 mM 3OMG in the presence of 10^{-6} M (solid circles) or 10^{-5} M (triangles) of CB. Each data point represents single determination. These results were reproduced in two additional experiments.

4. Discussion

GLUT1 has been the only facilitative glucose transporter isoform available as a pure and functional protein suitable for the detailed biochemical and structural elucidation. Here we describe a partial purification of GLUT2, another important GLUT isoform, and its functional reconstitution into vesicles. Our data show that the partially purified GLUT2 of rat hepatocytes catalyzes glucose transport with a high K_m and binds to and is inhibited by cytochalasin B with a low affinity, and indicate that these

GLUT2 characteristics seen in cells are indeed the properties of GLUT2 protein itself. The purified GLUT2 will be useful for direct biochemical and structural elucidation of this isoform as a high K_m glucose transporter as well as a putative glucose-sensor; these properties are unique to GLUT2 and therefore cannot be studied with purified GLUT1.

Gene sequence data indicate that GLUT2 [4,5] is a slightly larger protein than GLUT1 [3,8] with 55% amino acid sequence identity and similar transmembrane topology. A notable difference between these two isoforms is that the exoplasmic, glycosylated loop connecting the transmembrane helices 1 and 2 is much longer in GLUT2 than in GLUT1 (64 versus 32 amino acids, respectively). Significantly, however, rat liver GLUT2 is only 82% identical in amino acid sequence to human liver GLUT2, with the divergence heavily localized at this glycosylated exoplasmic loop. This large species difference of liver GLUT2 is in contrast to GLUT1 that is 97% identical between rat and human. On the other hand, hepatocyte GLUT2 and pancreatic β-cell GLUT2 are identical in amino acid sequence [11,42]. In Western blots, however, GLUT2 in liver and the β-cells show different electrophoretic mobilities migrating as 51- and 55-kDa polypeptides, respectively, suggesting that GLUT2 undergoes tissue-specific, post-translational modification. Our purified liver GLUT2 may also be useful in studying the molecular aspects of this modification.

There are several potential phosphorylation sites in GLUT2 sequences that may regulate its function. These include serines 489, 501 and 503, and threonine 510 (all in rat sequence) in the C-terminal cytoplasmic domain that were phosphorylated by the cyclic AMP-dependent kinase in pancreatic β-cells after activation of adenylyl cyclase causing reduction in the rate of 3OMG uptake [43]. The threonine is unique to rat sequence, whereas the three serines are conserved between rat and human GLUT2 sequences [6]. GLUT2 sequence also includes potential phosphorylation sites for the protein kinase C whose functional significance is currently unknown.

The chemical purity of our purified GLUT2 is difficult to assess directly. Protein staining of SDS-PAGE of the purified GLUT2 preparation (Fig. 4B) shows significant impurities, whose quantitation relative to GLUT2 protein mass was not possible because of poor stainability of the latter. Calculation based on the observed specific GLUT2 immunoreactivity (Table 2), however, gave approximately 2700-fold purification over the total membrane fraction. Specific cytochalasin B binding activity (8.4 nmol/mg protein), on the other hand, indicates that our purified GLUT2 is approximately 60% pure, assuming one-to-one binding stoichiometry.

Our data also indicate that the purified GLUT2 reconstituted in lipid vesicles is functional in that it binds cytochalasin B and catalyzes a mercury chloride-sensitive and cytochalasin B-inhibitable 3OMG transport. This functional reconstitution of GLUT2, however, appears to require stringent experimental conditions; it was so far possible only with OG and subsequent salt elution in DEAE chromatography (Figs. 4–6). Non-competitive inhibition of 3OMG flux by cytochalasin B (Fig. 6) and D-glucose-insensitive cytochalasin B binding (Fig. 5) observed here with purified GLUT2 in vesicles are strikingly different from competitive inhibition of the flux by cytochalasin B and D-glucose-displaceable cytochalasin B binding displayed by purified GLUT1 in vesicles [13,34], and emphasize fundamental differences in physicochemical characteristics between these two proteins.

Detergent solubility of liver GLUT2 is quite different from that of erythrocyte GLUT1. Most of the detergents that solubilized erythrocyte GLUT1, including OG, did not solubilize GLUT2 (Table 1 and Fig. 2). Of many non-ionic detergents tested, only W-1 and DM solubilized GLUT2 without aggregation. Furthermore, DEAE chromatographic behavior of GLUT2 in these detergents is complex. The time course of salt elution in DEAE chromatography of GLUT2 in DM (Fig. 3B) suggests that the GLUT2 eluted in these two peaks represent two distinct physicochemical states of GLUT2 rather than a chromatographic artifact. A significant portion of GLUT2 immunoreactivity occurs in an endoplasmic reticulum enriched microsomal fraction free of plasma membrane markers (Lachaal et al., unpublished data). This suggests the presence of two subpopulations of GLUT2 in hepatocytes, one at the plasma membrane and the other in the endoplasmic reticulum, which may be related to the two distinct physicochemical behaviors of GLUT2 in DEAE chromatography observed here.

Functional reconstitution of purified GLUT2 was successful after chromatography in OG (Fig. 4B). For DM-solubilized GLUT2, extraction with anhydrous acetone at dry ice temperature removed DM in the presence of lipids and sodium cholate without aggregation or loss of GLUT2 as revealed by SDS–PAGE immunoblots (not illustrated). Dialysis of this sodium cholate solution of protein residue and lipids [41,44] gave reconstitution of GLUT2 into vesicles, but failed to demonstrate CB binding or 3OMG transport activities (not illustrated).

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